IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re Application of:	) For:	Enhanced Herbicide Composition
	)	
Silverman, et al.	)	
	)	
Serial No.: 10/619,347	) Group Art Unit:	1616
	)	
Filed: July 14, 2003	) Examiner:	S. Clardy

DECLARATION UNDER 37 C.F.R. §1.132

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Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313

Sir:

1. I, F. Paul Silverman, am an inventor of this application.

2. I have been employed by Valent BioSciences Corporation, the assignee of this application, since January 2000.

3. I currently hold the position of Senior Scientist.

4. I have read and understand this application including the claims, the Office Action dated July 6, 2004 and the cited and applied prior art.

5. The following experiments were performed by me or under my supervision and show that salicylate potentiation of PSII in inhibiting herbicides neither works through NO_x nor is it SAR dependent. Klepper (1988. Pest. Biochem. Physiol. **32**:173) investigated the hypothesis that NOx production is causal to herbicide action, and that the combination of PSII inhibitors and SA could increase the production of NOx by plants.

Nitrogen oxides, or NOx, is a term for a group of reactive gases, that contain nitrogen and oxygen in varying amounts. In plants, NO, or nitric oxide is the primary gas produced (see Klepper, p 174, second line). NO has been identified over the last decade as physiologically important to plants. Klessig et al. (2000, PNAS USA **97**:8849) highlighted the role of NO in the plant defense response. The recent cloning of the NO synthase (NOS) underscored the role of NO in ABA-regulation of

stomatal closure (Guo et al. 2003, Science **303**:100). Moreover, NO has recently been shown to be involved in regulation of time of flowering (He et al., 2004. Science **305**:1968). Thus NO has been shown to be a signal molecule in regulation of several aspects of plant development. Work with NO has been assisted by the development of NO-releasing chemicals. One of these, sodium nitroprusside (SNP) readily decomposes to yield NO. We used SNP to test whether SA potentiation of atrazine is coupled to NO. The results are shown in Table 1.

In the experiment conducted, both NO and salicylate were able to potentiate atrazine herbicidal activity on tobacco (Table 1). However, NO completely inhibited salicylate potentiation at both 48 and 72 hours post spraying. These results suggest that salicylate and NO are antagonistic, as is seen in animal systems. In mammalian cells, salicylates have been reported to be efficient scavengers of NO (Hermann et al. 1999. FEBS Let. **445**:212), and salicylates inhibited the transcription of NOS2 (Farivar et al., 1996. JBC **271**:31585).

In plants, the relationship between NO and salicylate is less well understood. However, NO appears to function upstream of salicylate in plant defense, and may inhibit both catalase and ascorbate peroxidase (Clark et al., 2000. MPMI **13**:1380), which may regulate cellular redox state. Interestingly, SA has been postulated to also inhibit these enzymes as part of its function (Durner et al., 1995. PNAS USA **92**:11312; Durner et al., 1996. JBC **271**:28492). Thus, NO and SA may compete for some molecular targets. In order to minimize competition, both NaSA and the SNP were used at the same molarity (5 mM) in our studies.

Table 1. Effect of the NO generator sodium nitroprusside (SNP) on sodium salicylate (NaSA) potentiation of atrazine herbicidal activity on tobacco.

Treatments	48h post spraying	72h post spraying
	Percent leaf area Damaged	
Crop Oil Concentrate, 0.25% (v/v)	0 A	0 A
NaSA, 800 mg/l + COC 0.25%	1.1 A	1.1 A
SNP, 1490 mg/l (5 mM) + COC 0.25%	2.0 A	2.5 A
NaSA, 800 mg/l + SNP, 1490 mg/l + COC 0.25%	3.5 A	4.5 A
Atrazine, 250 mg/l + COC 0.25%	51.5 B	79.8 B
Atrazine, 250 mg/l + NaSA, 800 mg/l + COC 0.25%	73.5 C	93.8 D
Atrazine, 250 mg/l + SNP, 1490 mg/ l + COC 0.25%	62.5 BC	82.8 BC
Atrazine, 250 mg/l + NaSA, 800 mg/l + SNP, 1490 mg/l COC 0.25%	47.0 B	81.3 B

n = 4 plants. Mean separation by Duncan's New Multiple Range Test ($\alpha = 0.05$).

In these studies, NO clearly inhibited salicylate potentiation of atrazine, indicating a separate and distinct mechanism for salicylate activation of herbicidal activity.

Klepper (1988) suggested that NOx was key to atrazine activity and that salicylate increased NOx production. However, we showed in the data above that NO in fact inhibits rather than increases salicylate potentiation of atrazine. Thus, SA potentiation of atrazine activity is independent of NO.

Ryals et al. (1996. Plant Cell **8**:1809) discloses that potentiation of atrazine activity is observed in response to sodium salicylate, acibenzolar-S-methyl, and other substituted salicylates.

Potentiation of PS II inhibitors was observed in response to salicylate, acibenzolar-S-methyl, and other substituted salicylates. Although the activity of the selected SAR inducers correlates with their ability to potentiate atrazine, this relationship is not necessarily causal. The study of the role of SA in SAR has been aided through the use of mutants that are modified in their response to salicylate. To assess the role of SAR in salicylate potentiation of atrazine activity, we used the *npr1* mutant of Arabidopsis. The NPR gene encodes a transcriptional regulator, which controls the ability of salicylate and some SAR inducers, to induce pathogenesis-related proteins and immunity to disease (Cao et al. 1997. Cell **88**:57). In *npr1*plants, salicylate is unable to induce PR proteins and disease resistance.

Treatment of *npr1* plants resulted in salicylate potentiaition of atrazine on these plants equal to what was observed in the Nossen wild type. This result is shown in Table 2.
Analysis of these data suggests that SA-induced SAR is not necessary for SA-potentiation of atrazine. In fact, the results with the *npr* mutant demonstrate that these phenomenon are separable.

Table 2. Effect of sodium salicylate (NaSA) on atrazine herbicidal activity on Nossen or *npr1-5* Arabidopsis

Treatments	Nossen (WT)	*npr1-5*
Phytotoxicity at 2 day after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	12.5 B	7.8 A
Atrazine 100 mg/l + COC 0.1%	0 A	0 A
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	34.8 CD	23.1 B
Phytotoxicity at 5 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	13.5 B	6.5 A
Atrazine 100 mg/l + COC 0.1%	26.8 C	14 A
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	58.5 D	51.5 B
Phytotoxicity at 7 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	10.8 B	9.5 A
Atrazine 100 mg/l + COC 0.1%	41.5 C	39.0 B
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	81.3 D	74.0 C
Phytotoxicity at 12 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	9.0 B	7.8 A
Atrazine 100 mg/l + COC 0.1%	85.3 C	93.8 B
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	98.8 D	96.9 B

n = 4 plants. Mean separation by Duncan's New Multiple Range Test ($\alpha = 0.05$).

Studies with the ethylene insensitive mutant *ein2-1* demonstrate that jasmonate/ethylene-dependent induced systemic resistance (ISR) is not required for salicylate-induced atrazine potentiation (Table 3).

Table 3. Effect of sodium salicylate (NaSA) on atarazine herbicidal activity on Columbia or *ein2-1* Arabidopsis		
Treatments	**Nossen (WT)**	***ein2-1***
Phytotoxicity at 2 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	6.3 AB	12.5 BC
Atrazine 50 mg/l + COC 0.1%	0.8 A	4.5 AB
Atrazine 50 mg/l + 400 mg/l NaSA + COC 0.1%	68.8 C	76.5 D
Phytotoxicity at 6 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	7.8 B	14.0 B
Atrazine 50 mg/l + COC 0.1%	42.3 C	51.5 C
Atrazine 50 mg/l + 400 mg/l NaSA + COC 0.1%	87.5 D	84.5 D
Phytotoxicity at 9 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	7.8 A	7.8 B
Atrazine 50 mg/l + COC 0.1%	67.8 B	87.5 C
Atrazine 50 mg/l + 400 mg/l NaSA + COC 0.1%	98.8 C	95.0 D
n = 4 plants. Mean separation by Duncan's New Multiple Range Test ($\alpha = 0.05$).		

In addition to its role in SAR, salicylate has several other physiological roles in plants. SA has been shown to be the trigger for thermogenesis in some Arum lilies, an inducer of alternative oxidase in non-overtly thermogenic plants, and it may also regulate ion channels (see Raskin, I. 1992. Ann.Rev. Plant Phys. Plant Mol.Biol. **43**: 439). As such, salicylate has many independent roles in plants that may not be linked through the same signal transduction pathways. For example, SA may induce chilling resistance in rice (Kang, et al. 2002. Physiol.Plant. **15**: 571), in a plant where it does not induce SAR (Silverman, et al. (1995) Plant Phys.**108**: 633). We postulate that the effect of SA and SAR inducers on potentiation of PS II inhibiting herbicides is similar: although these compounds are inducers of disease resistance, potentiation of atrazine works through a yet-to-be defined pathway. The evidence for the pathway is the a) uncoupling of salicylate potentiation from NO, and b) the optional need for a functional SAR pathway.

Although these molecules are inducers of the plant defense response, we have shown in the experimental data that salicylate potentiation of atrazine is independent from SAR. The Arabidopsis *npr1* mutant, which is unable to induce defense-related proteins in response to salicylate or pathogens, shows synergy between atrazine and salicylate. Due to the *npr1* mutation, we must consider that salicylate potentiation of atrazine is not functioning through SAR. Therefore, the potentiation at atrazine by acibenzolar and other salicylates is not SAR-dependent. Thus, neither salicylate not acibenzolar potentiation of atrazine works through SAR.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that wilful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

By F. Paul Silverman

F. Paul Silverman

Date: December 23, 2004